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B7h, a Novel Costimulatory Homolog of B7.1 and B7.2, Is Induced by TNFα

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Summary

In a screen to identify genes induced by NF-κB/Rel transcription factors, we cloned a novel gene, *b7h*, that is a close homolog of B7 costimulatory ligands expressed on antigen-presenting cells. B7h can costimulate proliferation of purified T cells through a receptor on T cells distinct from CD28 or CTLA-4. Surprisingly, although B7h is expressed in unstimulated B cells, its expression is induced in both 3T3 cells and embryonic fibroblasts treated with TNFα, and it is upregulated in nonlymphoid tissues of mice treated with LPS, a potent activator of TNFα. These data define a novel costimulatory ligand for T cells and suggest that induction of B7h by TNFα may function as a mechanism to directly augment recognition of self during inflammation.

Introduction

NF-κB/Rel transcription factors are key regulators of proinflammatory genes (for review see Baeuerle, 1998; Ghosh et al., 1998; Sha, 1998). Because NF-κB/Rel transcription factors are uniquely positioned downstream of multiple innate and adaptive signaling pathways, they seem ideally placed to integrate and coordinate innate and adaptive signals required for formation of productive immune responses. In an effort to explore how this integration might be regulated by these transcription factors, we initiated a screen to identify NF-κB/Rel-dependent genes that are induced by the inflammatory cytokine TNFα.

TNFα is a potent inflammatory cytokine secreted principally by macrophages, but also by activated $CD4^+$ T cells, that has been implicated in the pathogenesis of a number of autoimmune conditions (for review see Vassalli, 1992; Feldmann et al., 1998). TNFα is both transcriptionally activated by NF-κB/Rel factors and leads to activation of these transcription factors through TNFα receptors (Collart et al., 1990; Shakhov et al., 1990; Hsu et al., 1995). This intimate relationship between TNFα and NF-κB/Rel transcription factors is highlighted by the recent demonstration that the embryonic lethality observed in $RelA^{-/-}$ mice is rescued by crosses to $TNF\alpha^{-/-}$ mice (Doi et al., 1999). TNFα is known to induce many gene products involved in inflammation, tissue repair, immune response, apoptosis, and hematopoiesis (Fiers, 1993). In an effort to identify TNFα-induced genes that were specifically regulated by NF-κB/Rel transcription factors, we initiated a subtractive screen using 3T3 cell lines generated from wild-type and $RelA^{-/-}$ embryonic fibroblasts (Beg and Baltimore, 1996). Surprisingly, one of the novel genes we identified was a close homolog of the B7.1 and B7.2 costimulatory ligands expressed on antigen-presenting cells.

Generation of an effective immune response by T cells requires at least two signals from antigen-presenting cells: one mediated by specific antigen bound to MHC molecules and a second antigen-independent signal mediated by costimulatory ligands (for review see Lenschow et al., 1996; Chambers and Allison, 1999). The primary costimulatory receptor expressed on T cells is CD28. Interaction of CD28 with either of its ligands, B7.1 or B7.2, results in enhanced T cell proliferation and cytokine secretion. Interactions of either B7.1 or B7.2 with CTLA-4, a homolog of CD28 expressed on T cells, results in inhibition of T cell responses (Walunas et al., 1994; Krummel and Allison, 1995). Recently, a third costimulatory receptor, ICOS, was identified that is a close structural homolog of CD28 and CTLA-4 (Hutloff et al., 1999). ICOS is induced on activated T cells and can costimulate T cell proliferation and a different spectrum of T cell cytokine production, but it does not appear to act through binding to B7.1 or B7.2.

B7.1 and B7.2 are related immunoglobulin supergene family members that are expressed by multiple cell types involved in antigen presentation (for review see McAdam et al., 1998). Both B7.1 and B7.2 are constitutively expressed on dendritic cells and are upregulated on monocytes, macrophages, B cells, and T cells following activation (Hathcock et al., 1994; Inaba et al., 1994). The upregulation on cells activated by various stimuli differs in terms of both the kinetics and density of expression of B7.1 and B7.2. B cells and monocytes, for example, upregulate B7.2 expression within 24 hr following activation with LPS, whereas B7.1 can only be detected 48 hr after activation, with the maximal cell surface expression being much less than observed with B7.2 (Freedman et al., 1991; Lenschow et al., 1993; Hathcock et al., 1994).

The complex regulation of B7.1 and B7.2 expression is consistent with these costimulatory ligands functioning as critical regulatory molecules for control of immune responses and has spurred considerable investigation of how the costimulatory environment may be altered or augmented during infection, inflammation, and autoimmune conditions. We report here the identification of a novel T cell costimulatory ligand, B7h, that is a third member of the B7 family based on its amino acid homology to B7.1 and B7.2. B7h interacts with a receptor on activated T cells distinct from CD28 or CTLA-4, and it costimulates proliferation of T cells. B7h is expressed in B cells and is most highly expressed in peripheral lymphoid tissues of mice. Surprisingly, B7h expression was induced in 3T3 cells and embryonic fibroblasts treated with TNFα and was upregulated in nonlymphoid tissues of mice injected with LPS, a potent inducer of TNFα (Beutler et al., 1986; Kornbluth and Edgington,

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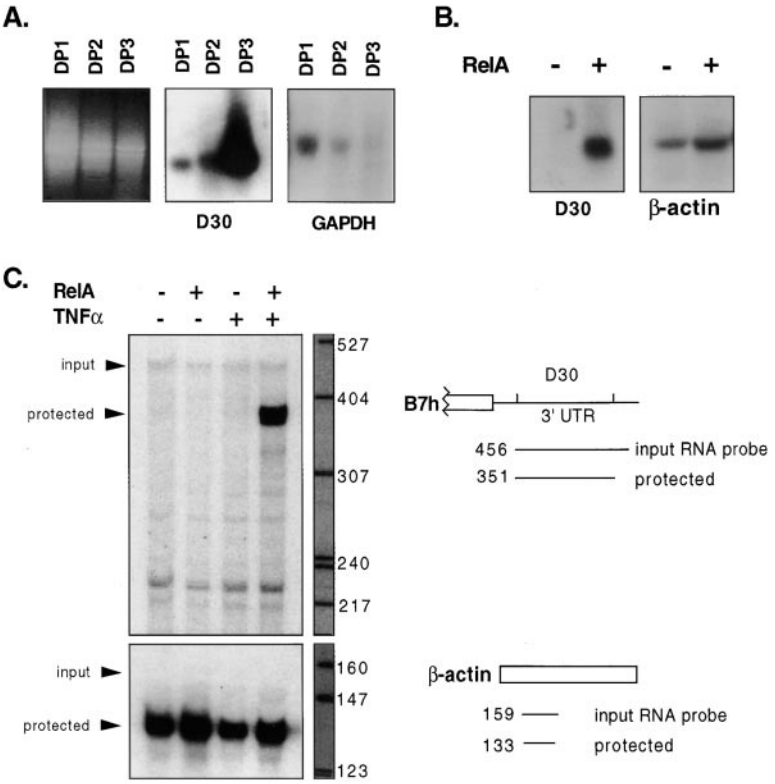

Figure 1. The D30 cDNA Fragment Obtained by RDA Corresponds to a Gene that Is Induced by TNFα and Dependent on RelA Activation

RDA was performed using polyA$^+$ RNA isolated from RelA$^{+/+}$ and RelA$^{-/-}$ 3T3 cells that were treated with TNFα (10 ng/ml) for 1.5 hr. RelA$^{+/+}$ 3T3 cells were used as the tester population in order to isolate cDNAs that were dependent on RelA activation.

(A) D30 is successively enriched after each round of RDA selection in the difference products DP1, DP2, and DP3. Difference products were electrophoresed on an agarose gel, transferred to a Hybond-N$^+$ membrane (Amersham), and hybridized with either a D30 or control GAPDH probe.

(B) D30 is expressed in cDNA prepared from RelA$^{+/+}$ but not RelA$^{-/-}$ 3T3 cells. Equivalent amounts of the original cDNA representations from 3T3 cells treated with TNFα for 1.5 hr were blotted and hybridized with either a D30 or control β-actin probe.

(C) D30 corresponds to a gene that is induced by TNFα and dependent on RelA for expression. Total RNA (15 μg) prepared from 3T3 cells was analyzed for D30 expression using RNase protection probes for D30 and β-actin.

1986). These data suggest a distinct role for B7h as a costimulatory ligand that can be transiently induced on non-antigen-presenting cells by inflammatory cytokines. Thus, induction of B7h by TNFα may function as a direct mechanism by which recognition of self by T cells is enhanced in local tissues during an inflammatory response.

Results

Identification of a TNFα-Induced Gene Dependent on NF-κB/Rel Activation

To identify TNFα-induced genes regulated by the RelA transcription factor, we performed cDNA subtraction by representational difference analysis (RDA) (Lisitsyn and Wigler, 1993; Hubank and Schatz, 1994). RDA was performed using TNFα-treated 3T3 cells that were derived from RelA$^{-/-}$ and wild-type RelA$^{+/+}$ embryonic fibroblasts (Beg and Baltimore, 1996). Wild-type 3T3 cells were used to prepare tester representations that were subtracted with an excess of driver representations prepared from RelA$^{-/-}$ 3T3 cells. After three rounds of enrichment for cDNA fragments that were differentially expressed in wild-type 3T3 cells, the third difference product was subcloned and individual cDNA enzyme fragments were characterized. One of these cDNA fragments, D30, did not correspond to a known gene or expressed sequence tag, was successively enriched in difference products 1, 2, and 3 (Figure 1A), and was expressed in the original representation from wild-type, but not RelA$^{-/-}$, 3T3 cells (Figure 1B).

Using an RNase protection probe corresponding to the D30 cDNA fragment, we examined expression of this candidate gene in RNA samples prepared from 3T3 cells before and after treatment with TNFα (Figure 1C). In both untreated wild-type and RelA$^{-/-}$ 3T3 cells, the gene corresponding to the D30 cDNA fragment was not expressed in the absence of TNFα. When these 3T3 cells were treated with TNFα, transcription was induced in wild-type 3T3 cells but not in RelA$^{-/-}$ 3T3 cells. These results demonstrate that the D30 cDNA fragment corresponded to a gene that was not normally expressed in 3T3 cells, but which could be induced by TNFα treatment, and required activation of the RelA member of the NF-κB/Rel transcription factor family for expression.

B7h Encodes for a Protein with Homology to B7 Costimulatory Ligands

Because the D30 cDNA fragment did not correspond to a previously identified gene, we screened a murine thymus cDNA library with a D30 probe and isolated a 2.7 kb cDNA clone. This clone size was consistent with the single mRNA band of approximately 2.8 kb that we observed in Northern blots of mRNA isolated from TNFα-treated 3T3 cells and from spleen tissue that was probed with the D30 cDNA fragment (data not shown). Sequence analysis of the 2.7 kb cDNA clone revealed that the 5′ end contained an open reading frame for a 322 amino acid protein that we termed B7h, for B7 homologous protein (Figure 2).

The *b7h* cDNA encoded for a type I transmembrane protein that was an immunoglobulin superfamily member. The domain structure of B7h was homologous to

Figure 2. The D30 Fragment Corresponds to a cDNA Encoding a Protein, B7h, Homologous to B7 Costimulatory Ligands

DNA sequence of the *b7h* cDNA is shown with the coding region in bold. The location of the D30 cDNA fragment in the 3′-untranslated region is underlined. The location of a second RNase protection probe used in Figures 6 and 7 is underlined in the coding region.

B7 costimulatory ligands and contained a signal peptide, two extracellular immunoglobulin-like domains, a transmembrane region, and a short cytoplasmic tail (Figure 3). Numerous potential N-linked glycosylation sites were identified in the extracellular portion of B7h, consistent with it being a transmembrane protein. The cysteine residues that are conserved in both the V- and C-like immunoglobulin domains of B7.1 and B7.2 were also conserved in B7h. The amino acid identities between B7h and B7.2 and between B7h and B7.1 were both 20%. Since B7.1 and B7.2 possess the same degree of amino acid identity, B7h is a B7 family member that is as closely related to B7.1 and B7.2 as these costimulatory ligands are to each other (Freeman et al., 1991, 1993).

B7h Interacts with Receptor(s) on Activated T Cells Distinct from CTLA4 or CD28

We confirmed that the *b7h* cDNA encoded for a transmembrane receptor by first examining the intracellular localization of a B7h-GFP fusion protein, generated by fusing the N terminus of green fluorescent protein (GFP) to the C terminus of the putative cytoplasmic domain of B7h. CHO cells were infected with retroviral vectors expressing either control GFP or the B7h-GFP fusion protein. When visualized by fluorescence microscopy, the B7h-GFP fusion protein was localized to the surface of cells, whereas the GFP expressed by control vector was seen distributed throughout cells (Figure 4A). To confirm that B7h was actually expressed on the cell

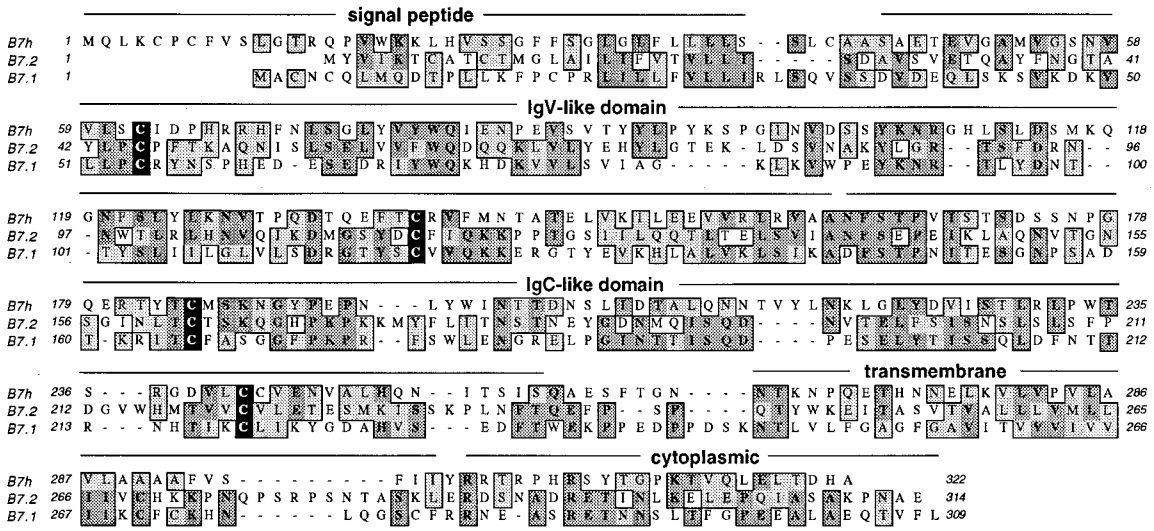

Figure 3. B7h Is a Member of the B7 Family of Costimulatory Ligands

Amino acid sequence alignment of B7h with murine B7.1 and B7.2 obtained using the Clustal-W algorithm with BLOSUM 30 matrix (MacVector, Oxford Molecular Group). Identical amino acid residues are shaded, conserved residues are boxed, and conserved cysteine residues are shown in black. Predicted signal peptide, Ig V-like and C-like domains, and the transmembrane region for B7h are indicated.

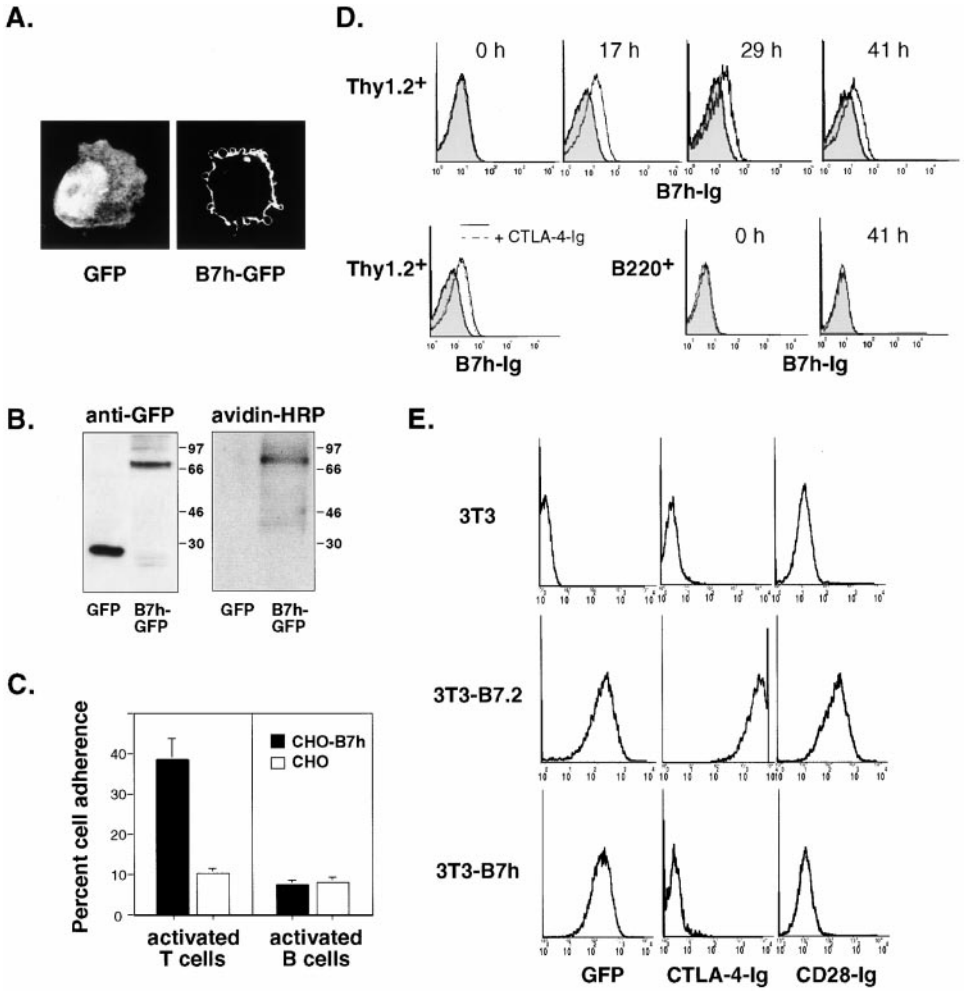

Figure 4. B7h Is a Transmembrane Ligand for a Receptor Expressed on Activated T Cells Distinct from CTLA-4 or CD28

(A) B7h localizes to the cell surface. CHO cells infected with retroviral vectors expressing either a B7h-GFP fusion protein or GFP alone were imaged by confocal fluorescence microscopy. GFP fluorescence is shown in white.

(B) B7h is a transmembrane protein. Whole cell lysates of CHO cells expressing either B7h-GFP or GFP were immunoblotted with an anti-GFP antibody. Intact CHO cells were cell surface labeled with biotin, lysed in 0.5% NP-40-containing buffer, immunoprecipitated with an anti-GFP antibody, and immunoblotted with streptavidin-HRP.

(C) Expression of B7h on CHO cells induces binding of activated T cells. Activated T and B cells labeled with ^{3}H-thymidine were added to 96-well plates containing confluent monolayers of CHO cells expressing either B7h or GFP. Plates were briefly spun and then incubated at 37°C for 45 min. Following five washes with PBS, ^{3}H-thymidine in wells was measured. Binding is presented as a percentage of input counts. Error bars indicate standard deviation of triplicate cultures.

(D) B7h-Ig binds to activated T cells. Resting and activated T and B cells were stained with a B7h-Ig fusion protein (~0.1 μg) and analyzed by flow cytometry. T cells were activated with PMA (10 ng/ml) and ionomycin (1 μg/ml) for the indicated times. B cells were activated with LPS (30 μg/ml). Specific staining of activated T cells was not inhibitable by addition of CTLA-4-Ig (5 μg). Control background staining is shown shaded in grey.

(E) B7h does not interact with CD28 or CTLA-4. 3T3 cells alone or expressing either B7.2 or B7h were stained with CTLA-4-Ig (1 μg) and CD28-Ig (2 μg) fusion proteins and analyzed by flow cytometry. Expression of B7.2 was monitored using an IRES-GFP marker, and expression of B7h was monitored using a B7h-GFP fusion protein.

surface, intact CHO cells expressing either GFP or B7h-GFP were biotinylated, and then lysates of these CHO cells were immunoprecipitated with an anti-GFP antibody. Immunoblotting of immunoprecipitates with streptavidin-HRP demonstrated that B7h-GFP was biotinylated, consistent with its localization by microscopy (Figure 4B). In contrast, intracellular GFP was not biotinylated but could be detected by immunoblotting whole cell lysates with an anti-GFP antibody. These results demonstrate that B7h is a transmembrane molecule expressed on the cell surface.

Given the striking homology between B7h and B7 costimulatory ligands, we next examined whether expression of B7h on CHO cells could result in formation of cell surface contacts with T cells. We observed that approximately 3- to 5-fold more activated T cells bound to CHO cells expressing B7h than to control CHO cells expressing GFP. To quantitate this binding of T cells, activated T cells labeled with ^{3}H-thymidine were plated on CHO cells, and then washed to remove nonadherent cells. A 4-fold greater number of activated T cells bound to CHO-B7h cells over CHO-GFP cells (Figure 4C). This

interaction was specific to activated T cells, as activated B cells demonstrated no preferential association with B7h expressing CHO cells. These results indicate that B7h is a membrane ligand for cell surface receptor(s) expressed on activated T cells.

To test whether the physical interaction between B7h and activated T cells occurred via CD28 or CTLA4 costimulatory receptors, we performed flow cytometric analysis of T cells using a B7h-Ig fusion protein generated by fusing the putative binding domains of B7h to the CH2-CH3 domains of mouse IgG1. B7h-Ig bound specifically to the surface of activated T cells but not to the surface of naive T cells or resting or activated B cells (Figure 4D). This binding was not blocked by addition of CTLA-4-Ig, indicating that B7h bound to a receptor induced on activated T cells distinct from CTLA-4 or CD28.

Further evidence that B7h does not interact with CD28 and CTLA-4 coreceptors on T cells came from reciprocal experiments in which bulk 3T3 cell lines expressing cell surface B7h were analyzed by flow cytometric analysis for binding to CTLA-4-Ig and CD28-Ig fusion proteins, comprised of the binding domains of CTLA-4 and CD28 linked to human immunoglobulin constant regions (Figure 4E). 3T3 cells, which normally do not express B7.1 or B7.2, were transduced with retroviral vectors for B7h and B7.2 at 100% efficiency with retroviral vectors, based on GFP marker expression. Because of the greater affinity of CTLA-4 for binding to B7 members (van der Merwe et al., 1997), 3T3 cells expressing B7.2 stained much more strongly with CTLA-4-Ig than with CD28-Ig (Figure 4E). In contrast, 3T3 cells expressing B7h did not show any specific staining with either CTLA-4-Ig or CD28-Ig. These results are consistent with the staining results with B7h-Ig that indicate that B7h does not bind or interact with either CD28 or CTLA-4 receptors. Thus, the interaction of B7h with activated T cells occurs by a receptor distinct from CD28 or CTLA-4.

B7h Functions as a Costimulatory Ligand for T Cells

Given the sequence homology to B7 costimulatory ligands and the interaction of B7h with activated T cells, we next examined whether B7h could function as a costimulatory ligand for T cells. We first tested whether B7h could costimulate T cell proliferation in *trans*, by examining the ability of 3T3 cells expressing either B7h, GFP, or B7.2 to costimulate proliferation of purified lymph node T cells in plates coated with different amounts of anti-TCR antibodies (Figure 5A). B7.2-expressing 3T3 cells resulted in costimulation of T cell proliferation at submitogenic doses of anti-TCR antibodies that did not result in proliferation of T cells incubated with GFP-expressing 3T3 cells. B7h-expressing 3T3 cells also costimulated T cell proliferation at submitogenic doses of anti-TCR antibodies, although the costimulatory effect required higher doses of anti-TCR antibodies than seen with B7.2-expresing 3T3 cells. Thus, B7h could costimulate proliferation of T cells in *trans*.

To test the ability of B7h to costimulate T cell proliferation in *cis*, purified transgenic T cells from DO11.10 transgenic TCR mice were plated on 3T3 cells expressing I-A^d alone or in combination with either B7h, B7.2, or GFP. Again we observed that B7h could costimulate proliferation of T cells at submitogenic doses of OVA peptide that induced no proliferation of T cells plated alone or on 3T3 cells expressing either I-A^d or I-A^d and GFP (Figure 5B). Addition of CTLA-4-Ig to block interactions between CD28 and B7 ligands had no effect on the ability of 3T3 cells expressing B7h to costimulate T cell proliferation under conditions in which costimulation by B7.2-expressing 3T3 cells was inhibited (Figure 5C). These data indicate that B7h costimulates T cell proliferation via a CD28-independent mechanism.

Specific Induction of B7h by TNFα

The observation that TNFα treatment of 3T3 cells resulted in expression of a costimulatory ligand for T cells prompted us to examine whether other cytokines or inflammatory stimuli could also induce B7h expression. Analysis using a second RNase protection probe corresponding to the coding region of B7h demonstrated that treatment of 3T3 cells with TNFα rapidly induced expression of B7h that was maximal at 16 hr and appeared self-limiting by 24 hr (Figure 6A). Treatment of 3T3 cells with IFNγ, however, did not lead to induction of B7h under conditions in which class I MHC was induced, nor did it synergize with TNFα in induction of B7h expression (Figure 6B). B7h was also not induced by either IL-1α or LPS treatment alone, indicating that the induction of B7h expression by TNFα was highly specific.

Although B7.1 and B7.2 are expressed in vivo on immune cells involved in antigen presentation, these molecules are constitutively expressed on certain cell lines, such as sublines of L cells, that were originally derived from primary cells of non-antigen-presenting origin. Consequently, we wanted to examine whether our observation that TNFα could induce B7h expression was a property peculiar to immortalized 3T3 cell lines. Using early passage embryonic fibroblasts prepared from day 12 embryos, we observed that these primary embryonic fibroblasts did not express B7h and that TNFα treatment induced expression of B7h in these cells (Figure 6C). This result indicates that induction of B7h expression by TNFα is a property of both primary embryonic fibroblasts and 3T3 cell lines derived from these fibroblasts.

LPS-Induced Expression of B7h in Nonlymphoid Tissues

To explore the potential physiological relevance of the ability of TNFα to induce B7h expression, the expression pattern of B7h was examined in tissues of normal mice and in tissues of mice challenged with LPS, a potent inducer of TNFα (Beutler et al., 1985; Mathison et al., 1988). In normal mice, B7h was most highly expressed in peripheral lymphoid tissues, spleen, and lymph node (Figure 7A). Constitutive expression of B7h was also observed in splenocytes and in B cells purified from spleen. Lower levels of B7h expression were found in many nonlymphoid tissues such as kidney, liver, peritoneum, and testes. Intermediate levels of B7h expression were observed in lung and thymus.

We next examined by an RNase protection assay whether B7h expression was induced in tissues of mice at different time points following intraperitoneal injection

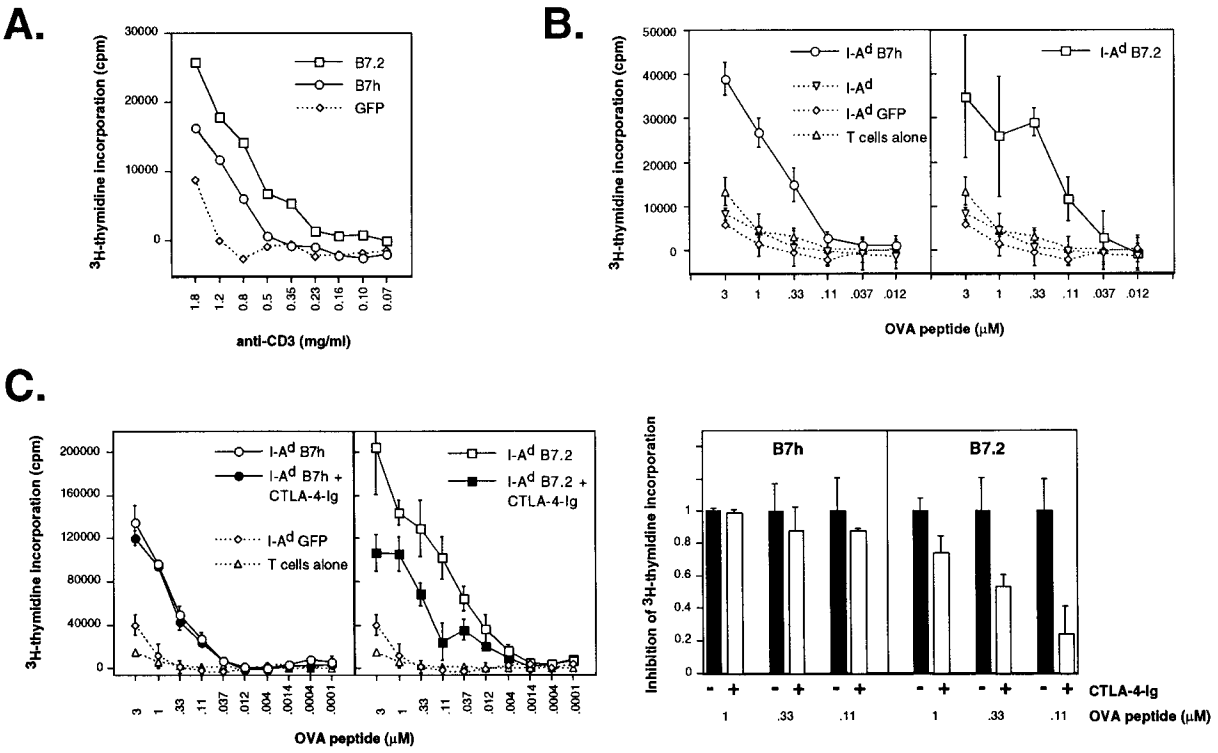

Figure 5. B7h Costimulates T Cell Proliferation

Proliferation was measured by ^{3}H-thymidine incorporation in cultures pulsed in the last 18 hr of a 72 hr incubation with ^{3}H-thymidine (1 μCi/well [6.7 Ci/mmol] in [A and B], 2 μCi/well [20 Ci/mmol] in [C]).

(A) Proliferation of lymph node T cells is costimulated in vitro by 3T3 cells expressing B7h. Purified T cells (10^5) were cultured in complete media on plates coated with the indicated concentrations of anti-CD3 (mAb 500A2) and 6×10^3 3T3 cells expressing either B7.2, B7h, or GFP. Proliferation of irradiated 3T3 cells in the absence of anti-CD3 has been subtracted from presented values, which represent averages of triplicate cultures that varied by no more than 35%.

(B) Proliferation of DO11.10 transgenic T cells is costimulated in vitro by 3T3 cells expressing I-A^d and B7h. Purified D011.10 transgenic T cells (5×10^4) were plated alone or on confluent 3T3 cells (12×10^3) expressing I-A^d alone or in combination with B7.2, B7h, or GFP. OVA peptide was added to cultures at the indicated concentrations. Proliferation of irradiated/mitomycin-C-treated 3T3 cells in the absence of peptide has been subtracted from presented values. Error bars indicate standard deviation of triplicate cultures.

(C) Costimulation of T cell proliferation by B7h is not dependent on interactions with CD28 or CTLA-4. Under conditions described in (B), CTLA-4-Ig (5 μg/ml) was added to purified D011.10 transgenic T cells plated alone or on confluent 3T3 cells expressing I-A^d with B7.2, B7h, or GFP. Data was treated as described in (B). Normalized data for inhibition of ^{3}H-thymidine incorporation by of CTLA-4-Ig is shown at three submitogenic concentrations of OVA peptide where both B7h and B7.2 costimulate T cell proliferation.

of LPS (Figure 7B). Of four nonlymphoid tissues examined, testes, kidney, and peritoneum showed induction of B7h expression that was maximal at 6 hr after LPS injection. The fourth nonlymphoid tissue, liver, showed no induction of B7h expression. In spleen, where B7h was basally expressed, a decrease in B7h expression was observed. This lack of induction of B7h expression in spleen was consistent with our observation that in vitro cultures of splenocytes treated with either TNFα or LPS did not result in induction of B7h expression (data not shown).

The induced expression of B7h in tissues of LPS-treated mice differed in two important respects from the expression pattern of B7.2 in the same tissues. First, in contrast to B7h expression, B7.2 expression was rapidly induced in the spleens of mice injected with LPS. This result was consistent with the ability of LPS to induce B7.2 expression in B cells (Hathcock et al., 1994; Inaba et al., 1994). Second, in nonlymphoid tissues where we observed clear induction of B7h expression, no induction of B7.2 expression was seen.

These results demonstrate that in an acute inflammatory response where TNFα is secreted, B7h was upregulated in nonlymphoid tissues. While we cannot identify the cellular source of LPS-induced B7h expression in these nonlymphoid tissues, it is unlikely that this increase in B7h expression arose from blood contamination, since no induction of B7h expression was observed in liver, a highly vascular tissue. Further, the maximal induced levels of B7h expression in nonlymphoid tissues were as high as 20% of the levels observed in the same amount of RNA from spleen. In these same tissues, the levels of B7.2 expression were not detectable relative to the levels observed in spleen.

Discussion

We have identified a novel T cell costimulatory ligand, B7h, that is a close homolog of the B7.1 and B7.2 costimulatory ligands expressed on antigen-presenting cells. This molecule costimulates T cell proliferation via a CD28-independent mechanism. It is expressed in B

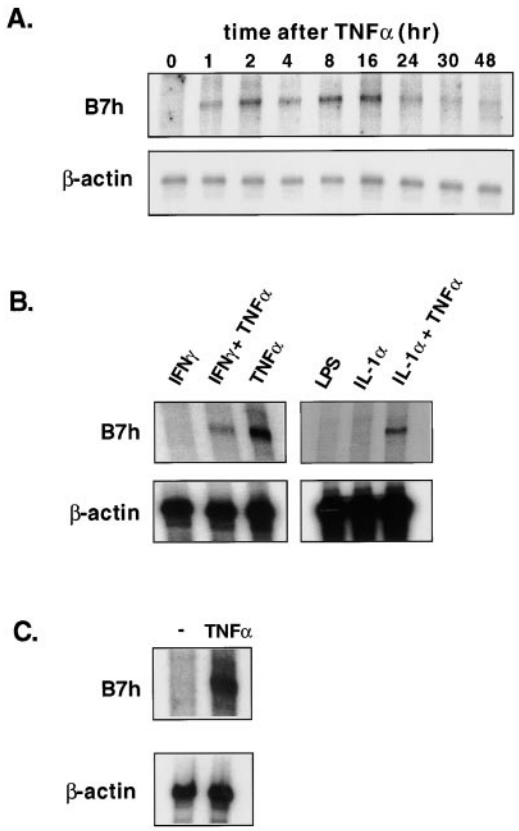

Figure 6. B7h Is Specifically Induced in 3T3 and Embryonic Fibroblasts by TNFα

Total cellular RNAs (15 μg) were prepared and analyzed for expression of B7h and β-actin by an RNase protection assay.

(A) 3T3 cells were treated with TNFα (10 ng/ml) and analyzed for B7h expression at the indicated time points.

(B) 3T3 cells were treated with IFNγ (50 U/ml) with and without TNFα (10 ng/ml) for 1.5 hr. Cell surface expression of class I MHC K^b was upregulated on 3T3 cells at the IFNγ concentrations used (data not shown). 3T3 cells were treated with IL-1α (10 ng/ml) or LPS (100 μg/ml) for 8 hr.

(C) Embryonic fibroblasts were treated with and without TNFα (10 ng/ml) for 4 hr.

cells, and in vivo it is highly expressed in peripheral lymphoid tissues. One candidate for the interacting partner of B7h on T cells is the recently described ICOS molecule that is a close homolog of CD28 and CTLA-4 (Hutloff et al., 1999). Because ICOS is expressed only on activated T cells, the ability of ICOS to costimulate T cell proliferation and cytokine release is more restricted than CD28, which is expressed on resting T cells. Thus, the costimulatory activity of ICOS is observed only above the threshold of T cell activation where ICOS becomes expressed. Our studies with B7h are consistent with a partner molecule with properties similar to ICOS, since B7h binds to a receptor on activated T cells and costimulates T cell proliferation in a narrower range of TCR activation than B7.2.

A striking property of B7h, which suggests a functional role quite distinct from B7.1 and B7.2, is its ability to be induced in 3T3 cells and embryonic fibroblasts in response to TNFα treatment. This cytokine-mediated induction of B7h expression by TNFα is rapid, highly specific, and dependent upon activation of RelA. Although B7.1 and B7.2 are largely confined to expression on antigen-presenting cells, it has been reported that expression of B7.1 but not B7.2 can be induced after treatment with a combination of IFNγ and TNFα using short-term fibroblast-like cell lines (FCLs) derived from pancreas, muscle, and skin (Pechhold et al., 1997). The induction of B7h we report here differs from the induction of B7.1 observed in short-term fibroblast cultures in several important respects. First, constitutive B7.1 expression was observed on most FCLs even in the absence of cytokines. In contrast, no expression of B7h was detected in primary embryonic fibroblasts or 3T3 cells in the absence of cytokines. Second, the upregulation of B7.1 expression occurred over several days, whereas the induction of B7h expression occurred rapidly within 1 hr after cytokine treatment. Finally, the upregulation of B7.1 expression required treatment with both IFNγ and TNFα. In contrast, the induction of B7h expression was induced by TNFα treatment alone, indicating that cytokine products of activated T cells or NK cells were not necessary for induction of B7h expression.

Ectopic expression of costimulatory ligands on non-antigen-presenting cells has been shown to result in heightened immune responses and, in some cases, in an increased propensity to develop autoimmunity. Immunization with tumor cells transfected with B7 ligands can result in antigen-specific T cell responses of sufficient magnitude to allow rejection of parental tumors upon rechallenge (Chen et al., 1992; Townsend and Allison, 1993). In transgenic models where B7 ligands were expressed on parenchymal cells of different tissues, animals had amplified host immune responses and in some models were more prone to developing local inflammation and frank autoimmunity (Guerder et al., 1988; Harlan et al., 1994; Nasir et al., 1994; Williams et al., 1994).

Our results demonstrating rapid induction of B7h expression in nonlymphoid tissues of mice treated with LPS are consistent with the induction of B7h expression we observed in TNFα-treated 3T3 cells and embryonic fibroblasts and indicate that inflammatory cytokines elicited in vivo can also induce B7h expression in a wide variety of tissues. While we cannot determine whether the induced B7h expression we observed resulted from parenchymal cells or from resident or recruited antigen-presenting cells, the differential upregulation of B7h in local tissues (but not spleen) was striking when compared to upregulation of B7.2, which was induced in spleen but was only detectable at very low levels in the nonlymphoid tissues examined. This differential regulation of B7h expression in response to LPS treatment suggests that induced B7h may play an important role in mediating local tissue responses to inflammatory conditions.

The ability of inflammatory cytokines to upregulate the local costimulatory environment has been reported in a variety of different experimental systems. For instance, fibroblasts transfected with viral proteins were capable of directly inducing antiviral cytotoxic T cell responses in vivo without involvement of host APCs, but only when the fibroblasts were placed in the cytokine-rich environment of lymphoid tissues (Kundig et

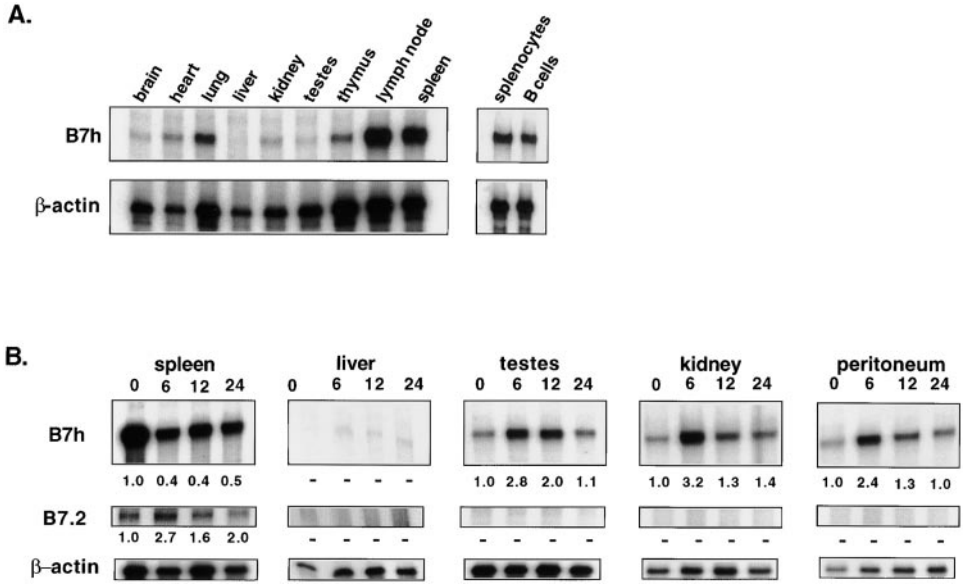

Figure 7. B7h Expression in Normal Mice and Mice Injected with LPS

Total cellular RNA (15 μg) prepared from tissues of normal mice or mice injected intraperitoneally with LPS were analyzed for B7h, B7.2, and β-actin expression by RNase protection analysis.

(A) B7h is highly expressed in peripheral lymphoid tissues of normal C57BL/6 mice and in splenocytes and purified splenic B cells.

(B) B7h expression is upregulated in nonlymphoid tissues of mice injected with LPS. (C57BL/6 × CBA)F_1 mice were injected intraperitoneally with 700 μg of LPS (Sigma) and tissues were harvested at 6, 12, and 24 hr timepoints following injection; controls not receiving LPS are shown as the 0 hr timepoint. Fold induction of B7h and B7.2 expression over the 0 hr timepoint for each tissue were quantitated by Phosphorimager and normalized to β-actin expression. Each RNA sample was simultaneously analyzed with a combination of three RNase protection probes for B7h, B7.2, and β-actin. Equivalent exposure times of autoradiographs for both B7h and B7.2 probes and tissues are shown; a shorter exposure time is shown for β-actin due to higher expression.

al., 1995). Local expression of B7.1 has been observed by RT–PCR and immunohistochemistry in lesions from tissues of patients with T cell–mediated inflammatory skin diseases and other autoimmune disorders (Simon et al., 1994; Windhagen et al., 1995; Imagawa et al., 1996).

Our identification of B7h as a costimulatory ligand that is constitutively expressed in B cells but is also capable of being induced by TNFα through activation of proinflammatory NF-κB/Rel factors suggests a simple mechanism that may contribute to how inflammatory cytokines augment immune responses. Given the higher threshold of TCR activation required for costimulation by B7h and the self-limited induction we report here, we speculate that one physiological role of B7h may be to augment immune responses in nonlymphoid tissues by costimulation of T cells after their primary activation in lymph nodes rather than by direct costimulation of naive T cells. Further work will be required to evaluate the in vivo significance of B7h as a costimulatory ligand in regulating normal immune responses and the extent and potential consequences of its upregulation by TNFα in inflammation and autoimmunity.

Experimental Procedures

Representational Difference Analysis

RDA was performed as described in Hubank and Schatz, 1994, and modified as described in Ouyang et al., 1999. $RelA^{+/+}$ and $RelA^{-/-}$ 3T3 cells (Beg and Baltimore, 1996) were treated with 10 ng/ml recombinant murine TNFα (Genzyme) and used to prepare tester and driver cDNA representations, respectively. After three rounds of RDA subtraction, DpnII-digested inserts were subcloned into a BamHI BSSKII(-) vector (Stratagene) and checked for differential expression against original cDNA representations. Positive clones were sequenced and RNase protection probes were used to confirm differential expression against RNAs prepared from 3T3 cells.

Cloning and Retroviruses

The *b7h* cDNA clone was isolated from a murine thymus cDNA library (Stratagene) and sequenced using an ABI Prism 310 Genetic Analyzer. A B7h-GFP fusion protein was created by subcloning a SalI–BamHI insert of B7h, generated by PCR using the oligonucleotides 5′-ACGCGTCGACCATGCAGCTAAAGTGTCCCTGTT-3′ and 5′-GGCGGATCCGGCGTGGTCTGTAAGTTCAAGCTG-3′, into the pEGFP-N3 mammalian expression vector (Clontech). A SalI–NotI insert corresponding to this B7h-GFP fusion protein was subcloned into a murine stem cell virus (MSCV) retroviral vector (Hawley et al., 1994). To create a B7h-Ig fusion protein, a second PCR insert of B7h was generated using the same 5′ oligonucleotide and one corresponding to the end of the C-like Ig domain, 5′-GGCGGATCCGCGT TATTTCCAGTGAAACTTTCTGCCTG-3′, and ligated in frame to the CH2-CH3 domain of mouse IgG1 at the Pro227 residue in the hinge region. This B7h-Ig insert was subcloned into a SalI–NotI pEGFP-N3 vector and transfected into Ig^- J558L cells. A murine *b7.2* cDNA was subcloned into a MSCV-IRES-GFP retroviral vector with a GFP marker expressed using an internal ribosomal entry sequence (Ranganath et al., 1998). An MSCV $A\alpha^d$-IRES-$A\beta^d$ retroviral vector (gift of Jim Allison) was used for transducing 3T3 cells with $I\text{-}A^d$. High-titer helper-free retroviral stocks were produced and used to infect cell lines as described (Pear et al., 1993). For infection of CHO cells, retroviral stocks were pseudotyped with vesicular stomatitis virus G-glycoprotein (VSV-G).

RNase Protection Assays

Total cellular RNAs were prepared from cell lines and tissues using Tri-Reagent (Molecular Research Center) according to manufacturer's protocol. Total RNA (15 μg) was used in each to analyze expression by RNase protection assays, which were performed as previously described (Sha et al., 1988). Two RNase protection probes

were used to examine B7h expression. The first B7h probe (Figure 1) corresponded to the original D30 DpnII insert (nucleotides 1309–1660) and the second B7h probe (Figures 6 and 7) corresponded to a PvuII–SmaI insert (nucleotides 243–591). Both of these inserts were subcloned into BSSKII(-) (Stratagene), and antisense RNA probes were generated using T7 RNA polymerase. The B7.2 RNase protection probe was generated by PCR from a murine *b7.2* cDNA using the oligonucleotides 5′-GGATCCTAATACGACTCACTATAG GGAGGTGAAATTGAGAGGTTTGGAGGAAAT-3′ and 5′-ACCGTAT CGAAGAGTCAAAATGGTCACCCGAAACCTAAGAAGATGTATT TTC-3′. For analysis of 3T3 cells and embryonic fibroblasts, 3×10^6 cells were plated overnight on 150 mm tissue culture plates. Cytokines were then added, and total cellular RNA was recovered from treated cells after washing with 1X PBS. Cytokines used were recombinant murine TNFα (Genzyme), recombinant murine IL-1α (R & D Systems), IFNγ (gift of Ken Murphy), and LPS (Sigma).

Immunofluorescence and Western Analyses

Fluorescent images were acquired on a Leica DMIRB/E fluorescence microscope and captured and processed using Slidebook software (Intelligent Imaging Innovations). Cell surface biotinylation of CHO cells and immunoprecipitations with an anti-GFP mAb (Quantum Biotechnologies) were performed as described in Lantz and Holmes, 1995. For Western analysis of immunoprecipitates, streptavidin-HRP (NEN) reactive bands were revealed by chemiluminescent detection (ECL; Amersham International). For Western blots of whole cell lysates, anti-GFP mAb was the primary antibody and goat anti-mouse Ig-HRP (Santa Cruz) was the secondary antibody.

Flow Cytometry

Culture supernatant or ascites from Ig^- J558L cells transfected with a B7h-Ig expression vector were used to stain cells with an anti-mouse-IgG PE-conjugate (Caltag) as a secondary reagent. Approximately 0.1 μg of B7h-Ig fusion protein was used to stain cells as quantitated by Western blot analysis using control mouse IgG1. Control background staining was determined using an equivalent amount of mouse IgG1 and normal mouse sera as a nonspecific primary control and the same secondary reagent. Bulk 3T3 cell lines were lifted from plates using 1X PBS with 1 mM EDTA and stained with 1 μg of purified CTLA-4-Ig (Linsley et al., 1991) and 2 μg of purified CD28-Ig (R & D Systems). A biotinylated anti-human-Ig antibody (Caltag) and a streptavidin-PE conjugate (Pharmingen) were used as secondary reagents. Cells were analyzed on a Coulter Epics XL.

Adherence and Costimulatory Assays

Highly purified T cells (>99.9% $Thy1^+$) were isolated from lymph node or from bulk cultures of resting D011.10 transgenic T cells (Murphy et al., 1990). Lymph node cells were first purified over nylon wool fiber columns (Polysciences), followed by lysis with guinea pig complement (GIBCO BRL) and rabbit complement (Cedarlane Lymph node cells in a cocktail of hybridoma supernatants [gift of Jim Allison] against HSA [J11.d], FcR [24G2], and anti-class II MHC [28.16.8S and BP107]). T cells were additionally purified by panning on plates coated with anti-mouse-Ig (Jackson ImmunoResearch). Splenocytes from D011.10 TCR transgenic mice were pulsed once with 0.3 μM OVA peptide (gift of Ken Murphy) and cultures were expanded as required in complete media. After 8–12 days, resting DO11.10 T cells were purified by nylon wool and complement lysis. Round-bottom plates (96-well) were coated with purified mAb 500A2 (gift of Jim Allison) at different concentrations, and 10^5 lymph node T cells were placed in each well with 6×10^3 3T3 cells irradiated with 6000R. For DO11.10 transgenic T cells, 5×10^4 T cells were placed in each well with 12×10^3 3T3 cells treated with mitomycin C and irradiated with 3000R. 3T3 cells used with D011.10 T cells expressed equivalent $I\text{-}A^d$ levels; by flow cytometry, (mean $I\text{-}A^d$ fluorescence)/(mean background fluorescence) was 4.90, 5.33, 5.05, and 4.26 for B7h, B7.2, GFP, and $I\text{-}A^d$ alone cells, respectively. Cells were labeled with ^{3}H-thymidine during the last 18 hr of a 72 hr incubation. Plates were harvested using an Inotech Harvester and ^{3}H-thymidine incorporation measured by gas ionization using a Packard Matrix 9600 Direct Beta Counter.

For adherence assays, activated T cells were labeled at day 3 by addition of ^{3}H-thymidine to cultures of peptide-stimulated DO11.10 spleen cells and used at day 4. Activated B cells were labeled at day 2 by addition of ^{3}H-thymidine to LPS-stimulated (30 μg/ml) C57BL/6 spleen cells and used at day 3. Cultures were purified over Histopaque-1119 (Sigma), and 10^5 cells were added to confluent CHO cells plated on 96-well flat-bottom plates.

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